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Limited Cross-Protection Provided by Prior Infection Contributes to High Prevalence of Influenza D Viruses in Cattle

Xiu-Feng Wan,[a,b,c,d,e,f] Lucas Ferguson,[f*] Justine Oliva,[g] Adam Rubrum,[h] Laura Eckard,[h] Xiaojian Zhang,[a,c,d] Amelia R. Woolums,[i] Adrien Lion,[g] Gilles Meyer,[g] Shin Murakami,[j] Wenjun Ma,[k] Taisuke Horimoto,[j] Richard Webby,[h] Mariette F. Ducatez,[g] William Epperson[i]

[a]Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, Missouri, USA
[b]Department of Electrical Engineering & Computer Science, College of Engineering, University of Missouri, Columbia, Missouri, USA
[c]Missouri University Center for Research on Influenza Systems Biology (CRISB), University of Missouri, Columbia, Missouri, USA
[d]Bond Life Sciences Center, University of Missouri, Columbia, Missouri, USA
[e]MU Informatics Institute, University of Missouri, Columbia, Missouri, USA
[f]Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi, USA
[g]IHAP, University of Toulouse, INRA, ENVT, Toulouse, France
[h]Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA
[i]Department of Population and Pathobiology Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi, USA
[j]Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
[k]Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas, USA

Xiu-Feng Wan and Lucas Ferguson contributed equally to this study. Author order was determined by a combination of manuscript contributions and seniority.

ABSTRACT Since its detection in swine, influenza D virus (IDV) has been shown to be present in multiple animal hosts, and bovines have been identified as its natural reservoir. However, it remains unclear how IDVs emerge, evolve, spread, and maintain in bovine populations. Through multiple years of virological and serological surveillance in a single order-buyer cattle facility in Mississippi, we showed consistently high seroprevalence of IDVs in cattle and recovered a total of 32 IDV isolates from both healthy and sick animals, including those with antibodies against IDV. Genomic analyses of these isolates along with those isolated from other areas showed that active genetic reassortment occurred in IDV and that five reassortants were identified in the Mississippian facility. Two antigenic groups were identified through antigenic cartography analyses for these 32 isolates and representative IDVs from other areas. Remarkably, existing antibodies could not protect cattle from experimental reinfection with IDV. Additional phenotypic analyses demonstrated variations in growth dynamics and pathogenesis in mice between viruses independent of genomic constellation. In summary, this study suggests that, in addition to epidemiological factors, the ineffectiveness of preexisting immunity and cocirculation of a diverse viral genetic pool could facilitate its high prevalence in animal populations.

IMPORTANCE Influenza D viruses (IDVs) are panzootic in multiple animal hosts, but the underlying mechanism is unclear. Through multiple years of surveillance in the same order-buyer cattle facility, 32 IDV isolates were recovered from both healthy and sick animals, including those with evident antibodies against IDV. Active reassortment occurred in the cattle within this facility and in those across other areas, and multiple reassortants cocirculated in animals. These isolates are shown with a large extent of phenotypic diversity in replication efficiency and pathogenesis but little in antigenic properties. Animal experiments demonstrated that existing antibodies could not protect cattle from experimental reinfection with IDV. This study suggests that, in addition to epidemiological factors, limited protection from preex-

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Address correspondence to Xiu-Feng Wan, wanx@missouri.edu.

* Present address: Lucas Ferguson, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California, USA.

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isting immunity against IDVs in cattle herds and cocirculation of a diverse viral genetic pool likely facilitate the high prevalence of IDVs in animal populations.

KEYWORDS influenza D virus, antigenic cartography, antigenic drift, cattle, evolution, genotype, humoral response, natural history, preexisiting immunity, protection

Influenza D virus (IDV) was first described as an influenza C-like virus after it was identified in a sick pig with influenza-like symptoms (1). IDV has a genome of seven segments, encoding polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase 3 (P3), hemagglutinin esterase fusion (HEF), nucleoprotein (NP), polyprotein P42, and nonstructural protein (NS) (2). Unlike influenza C viruses (ICV), IDV has a broad host range and cellular tropism, and a collection of virologic and serologic evidence suggests that IDV can infect domestic and feral swine, goats, sheep, camelids, buffalo, and equids; however, the primary host reservoir based on prevalence seems to be cattle (1–7). Along with a broad host range, IDV has been detected by either virologic or serologic surveillance across multiple continents, such as North America (the United States, Canada, and Mexico), Europe (e.g., France, Italy, Luxembourg, and Ireland), Asia (e.g., Japan and China), and Africa (e.g., Morocco, Togo, Benin, and Kenya) (1–3, 8–13). Evolutionary analyses suggested that IDV diverged from human ICV several hundred to several thousand years ago (14). Antigenic characterization using rabbit polyclonal sera on 10 IDVs from the United States suggested these IDVs can be grouped into two distinct antigenic groups (D/swine/Oklahoma/1334/2011 [D/OK]-like and D/bovine/Oklahoma/660/2013 [D/660]-like influenza viruses); genetic reassortment between viruses of these groups has been described (15, 16).

Virological surveillance indicated that IDVs can be recovered from both healthy and sick cattle, but the recovery rate in sick cattle is much higher (17). Etiologically, in North America, the cattle populations, especially sick cattle populations, at the order-buyer facilities seem to be a hotbed for IDV circulation (17). Under laboratory conditions, IDV can effectively infect and transmit between cattle, cause minor respiratory disease in cattle, and lead to neutrophil invasion in upper respiratory tract tissues (17, 18). In addition, IDV can experimentally infect pigs (1, 19), ferrets (1), guinea pigs (20), and mice (21); IDV can be robustly transmitted between cattle (17) but much less effectively in pigs, ferrets, and guinea pigs (1, 19, 20). The tissue tropisms of IDVs can vary depending on the animal models used, although typically, IDV was detected in the respiratory tract in cattle, pigs, ferrets, and guinea pigs (1, 16, 17, 19, 20) and in the respiratory tract, gastrointestinal tract, and even other organs (e.g., liver) in mice (21). Metagenomics studies suggested a strong correlation between the presence of IDV and a few other bovine respiratory disease (BRD) pathogens, leading to the assumption that IDV is one of the multifactorial pathogens involved in BRD (22, 23). However, experimental IDV challenge followed by *Mannheimia haemolytica* challenge did not increase the severity of clinical disease or pathology compared to that of cattle challenged with either agent alone (24).

Although there is clear evidence of wild spread of IDV in multiple host species, especially cattle, it is still largely unknown how IDV can circulate in cattle populations in the face of a high rate of seropositivity and limited evidence of viral antigenic variation. This study aimed to improve the understanding of how IDV emerges, evolves, and spreads through the use of multiple years of virological and serological surveillance in the same facility. We hypothesized that large diversities in antigenicity and other phenotypic properties among the IDVs would facilitate the emergence and spread of IDVs in bovine populations.

RESULTS

Serological and virological surveillance suggests IDVs are enzootic in cattle groups at an order-buyer facility. Between February 2014 and April 2016, 453 cattle from a single order-buyer facility were sampled in the hospital barn ($n = 134$) or upon

TABLE 1 Serological characterization of cattle sera collected from health cattle on arrival and sick cattle from the hospital at an order-buyer facility in Mississippi

		Serological characterization[d]					
Sampling time	*N*[c]	$N_{D/46N}$[e] (%)	$GMT_{D/46N}$[f] [min; max]	$N_{D/13N}$ (%)[g]	$GMT_{D/13N}$[h] [min; max]	$N_{D/46N\ or\ D/13N}$[i] (%)	$N_{D/46N\ and\ D/13N}$[j] (%)
Healthy cattle[a]							
March 2014	19	4 (21.05)	1:80 [1:40; 1:160]	10 (52.63)	1:70 [1:40; 1:640]	14 (73.68)	0 (0.00)
April 2014	37	8 (21.62)	1:73 [1:40; 1:160]	2 (5.41)	1:57 [1:40; 1:80]	8 (21.62)	2 (3.13)
May 2014	32	7 (21.88)	1:31 [1:40; 1:320]	11 (34.38)	1:80 [1:40; 1:160]	14 (43.75)	4 (12.50)
December 2015	29	14 (48.28)	1:69 [1:40; 1:160]	3 (10.34)	1:101 [1:40; 1:320]	16 (55.17)	1 (3.45)
January 2016	29	12 (41.40)	1:90 [1:40; 1:320]	1 (3.45)	1:40 [1:40; 1:40]	13 (44.83)	0 (0.00)
February 2016	30	1 (3.33)	1:80 [1:80; 1:80]	17 (56.67)	1:98 [1:40; 1:320]	18 (60.00)	0 (0.00)
March 2016	62	15 (24.19)	1:145 [1:40; 1:320]	5 (8.06)	1:70 [1:40; 1:80]	19 (30.65)	1 (1.61)
April 2016	81	22 (27.16)	1:68 [1:80; 1:160]	14 (17.28)	1:54 [1:40; 1:160]	33 (40.74)	3 (3.70)
Summary	319	83 (26.02)	1:86 [1:40; 1:320]	63 (19.75)	1:77 [1:40; 1:640]	135 (42.32)	11 (3.45)
Sick cattle[b]							
February 2014	45	12 (26.67)	1:160 [1:40; 1:1280]	12 (26.67)	1:57 [1:40; 1:160]	24 (53.33)	0 (0.00)
May 2014	11	7 (63.64)	1:177 [1:80; 1:640]	2 (18.18)	1:40 [1:40; 1:40]	8 (72.73)	1 (9.09)
January 2015	31	24 (77.42)	1:190 [1:40; 1:1280]	20 (64.52)	1:113 [1:40; 1:640]	25 (80.65)	19 (61.29)
July 2015	47	11 (23.40)	1:234 [1:40; 1:640]	10 (21.28)	1:184 [1:40; 1:320]	11 (23.40)	10 (21.28)
Summary	134	54 (40.30)	1:189 [1:40; 1:1280]	44 (32.84)	1:100 [1:40; 1:640]	68 (50.75)	30 (22.34)

[a]The 319 samples from the healthy cattle were collected upon arrival, excluding the 7 samples resampled from the groups.
[b]The 134 samples from the sick cattle.
[c]*N*, number of samples collected.
[d]Serological assays using HAI assays. A serum was determined to seropositive if the HAI titer was ≥1:40.
[e]$N_{D/46N}$, number of sample seropositive against D/46N; percent = $N_{D/46N}/N$.
[f]$GMT_{D/46N}$, geometric mean HAI titers for those that are ≥1:40 against D/46N; [min and max] represents the range of HAI titers between the lowest HAI titer and the highest HAI titer against D/46N.
[g]$N_{D/13N}$, number of samples seropositive against D/13N; percent = $N_{D/13N}/N$.
[h]$GMT_{D/13N}$, geometric mean HAI titers of ≥1:40 against D/13N; [min and max] represents the range of HAI titers between the lowest HAI titer and the highest HAI titer against D/13N.
[i]$N_{D/46N\ or\ D/13N}$, number of samples seropositive against either D/46N or D/13N; percent = $N_{D/46N\ or\ D/13N}/N$.
[j]$N_{D/46N\ and\ D/13N}$, number of samples seropositive against both D/46N and D/13N; percent = $N_{D/46N\ and\ D/13N}/N_{D/46N\ or\ D/13N}$.

arrival ($n = 319$). Serum samples were collected from the cattle at the hospital barn or the cattle upon arrival, and nasal swabs and/or nasopharyngeal swabs were from 78 sick cattle and 144 healthy cattle. The cattle upon arrival were typically healthy, whereas most of the cattle at the hospital barns showed BRD-like clinical signs (e.g., depression, low appetite, fever, nasal discharge, rapid/shallow breathing, and/or coughing).

To determine seroprevalence among these cattle, the sera were tested by hemagglutination inhibition (HAI) against D/bovine/Mississippi/C00013N/2014 (D/13N) (D/660-like virus) and D/bovine/Mississippi/C00046N/2014 (D/46N) (D/OK-like virus), two prototype viruses from two reported genetic clades (3, 25). The seroprevalence against either D/13N or D/46N was 42.32% in healthy cattle ($n = 319$) and 50.75% in sick cattle ($n = 134$) (Table 1). The positive HAI titers against D/13N and D/46N ranged from 1:40 to 1:1,280. In healthy cattle sampled from March 2014 to April 2016, seroprevalence ranged from 3.33% to 48.28% against D/46N, 3.45% to 56.67% against D/13N, and 21.62% to 73.68% against either D/46N or D/13N, whereas in sick cattle sampled from February 2014 to July 2015, seroprevalence ranged from 23.40% to 77.42% against D/46N, 21.28% to 64.52% against D/13N, and 23.40% to 80.65% against D/46N or D/13N.

Cattle sampled on arrival had an overall seroprevalence against either D/13N or D/46N of 42.32% ($n = 319$) (40.9% [$n = 88$] for 2014, 55.17% [$n = 29$] for 2015, and 41.09% [$n = 202$] for 2016) according to the sampling dates, and cattle in the hospital barn had seroprevalence against either D/13N or D/46N of 50.75% ($n = 134$) from 2014 to 2015 (57.14% [$n = 56$] for 2014 and 46.15% [$n = 78$] for 2015). Overall, the seroprevalence rates in the cattle from the hospital (sick cattle) were higher than those from the animals on arrival (healthy cattle) (Table 1), but no significant difference was identified between these two groups against D/46N ($P = 0.1535$), D/13N ($P = 0.2828$), or either D/46N or D/13N ($P = 0.4606$).

TABLE 2 Summary of the IDV isolates recovered from the same order-buyer cattle facility

Virus name[a]	Abbreviation	Sample date (mo/day/yr)	GenBank ID
D/bovine/Mississippi/C00002N/2014	D/2N	2/7/14	MT511416–MT511422
D/bovine/Mississippi/C00004N/2014	D/4N	2/19/14	MT511423–MT511429
D/bovine/Mississippi/C00007N/2014	D/7N	2/7/14	MT511409–MT511415
D/bovine/Mississippi/C00009P/2014	D/9P	2/7/14	MT511526–MT511532
D/bovine/Mississippi/C00013N/2014[b]	D/13N	2/7/14	MT511506–MT511511
D/bovine/Mississippi/C00013P/2014	D/13P	2/7/14	MT511347–MT511353
D/bovine/Mississippi/C00014N/2014[b]	D/14N	2/7/14	MT511444–MT511449
D/bovine/Mississippi/C00017N/2014	D/17N	2/7/14	MT511381–MT511387
D/bovine/Mississippi/C00020N/2014	D/20N	2/7/14	MT511457–MT511463
D/bovine/Mississippi/C00030P/2014[b]	D/30P	2/19/14	MT511368–MT511373
D/bovine/Mississippi/C00037P/2014	D/37P	2/19/14	MT511333–MT511546
D/bovine/Mississippi/C00040P/2014	D/40P	2/19/14	MT511388–MT511394
D/bovine/Mississippi/C00046N/2014[b]	D/46N	2/19/14	
D/bovine/Mississippi/C00067N/2014	D/67N	4/2/14	MT511478–MT511484
D/bovine/Mississippi/C00075N/2014	D/75N	4/2/14	MT511340–MT511346
D/bovine/Mississippi/C00135N/2014	D/135N	5/23/14	MT511361–MT511367
D/bovine/Mississippi/C00148N/2014	D/148N	1/9/15	MT511485–MT511491
D/bovine/Mississippi/C00158N/2015	D/158N	1/9/15	MT511450–MT511456
D/bovine/Mississippi/C00158P/2015	D/158P	1/9/15	MT511519–MT511525
D/bovine/Mississippi/C00162N/2015	D/162N	1/9/15	MT511492–MT511498
D/bovine/Mississippi/C00165N/2015	D/165N	1/17/15	MT511533–MT511539
D/bovine/Mississippi/C00165P/2015	D/165P	1/17/15	MT511471–MT511477
D/bovine/Mississippi/C00180N/2015	D/180N	7/24/15	MT511354–MT511360
D/bovine/Mississippi/C00185N/2015	D/185N	7/24/15	MT511402–MT511408
D/bovine/Mississippi/C00186N/2015	D/186N	7/24/15	MT511499–MT511505
D/bovine/Mississippi/C00196N/2015	D/196N	7/24/15	MT511374–MT511380
D/bovine/Mississippi/C00199N/2015	D/199N	7/24/15	MT511437–MT511443
D/bovine/Mississippi/C00200N/2015	D/200N	7/24/15	MT511395–MT511401
D/bovine/Mississippi/C00207N/2015	D/207N	7/24/15	MT511512–MT511518
D/bovine/Mississippi/C00213N/2015	D/213N	7/24/15	MT511430–MT511436
D/bovine/Mississippi/C00214N/2015	D/214N	7/24/15	MT511540–MT511546
D/bovine/Mississippi/C00218N/2015	D/218N	7/24/15	MT511464–MT511470

[a]The viruses with suffix N were recovered from nasal swabs and those with suffix P from nasopharyngeal swabs. These viruses also include three pairs of isolates, D/13N and D/13P, D/158N and D/158P, and D/162N and D/162P; each pair was recovered from the same animal but on different types of swabs.
[b]The HEF of D/13N, D/14N, and D/30P and the full genome of D/46N were reported by Ferguson et al. (3).

In total, 32 isolates were recovered from respiratory swabs, including 25 from nasal swabs (identified by the letter N) and 7 from nasopharyngeal swabs (identified by the letter P) (Table 2). These viruses include three pairs, D/13N and D/13P, D/158N and D/158P, and D/162N and D/162P, recovered from the same animal but from different types of swabs. The virus recovery rate from 78 individual sick cattle was 17.95% (14 cattle with 16 isolates), whereas that from 144 individual healthy cattle (15 cattle with 16 isolates) was only 10.41%.

Of interest, among these 30 cattle with recovered IDV isolates, only 13 were IDV seronegative. The remaining 16 cattle were shown to have an HAI titer of 1:10 or higher; one cattle had an HAI titer of 1:80 against D/13N and D/46N (see Table S1 in the supplemental material).

Genetic diversity of IDVs circulating in a single order-buyer facility. To facilitate our understanding of the genetic diversity of IDVs from the same order-buyer facility, we performed phylogenetic analyses on the HEF genes of the 32 isolates recovered from this study and 37 from the public databases. Phylogenetic analyses of HEF suggested that four genetic clades circulate worldwide. The viruses from the D/OK-like clade were from North America, Europe, and Asia, D/660-like was from North America and Europe, D/bovine/France/2986/2012 (D/Fra)-like was from Europe, and D/bovine/Yamagata/10710/2016 (D/Yam)-like was from Asia. The 32 isolates recovered from this study clustered with D/OK-like and D/660-like viruses (Fig. 1). The genetic diversities within each clade varied from 97.73% to 100% and from 95.09% to 96.96% between clades.

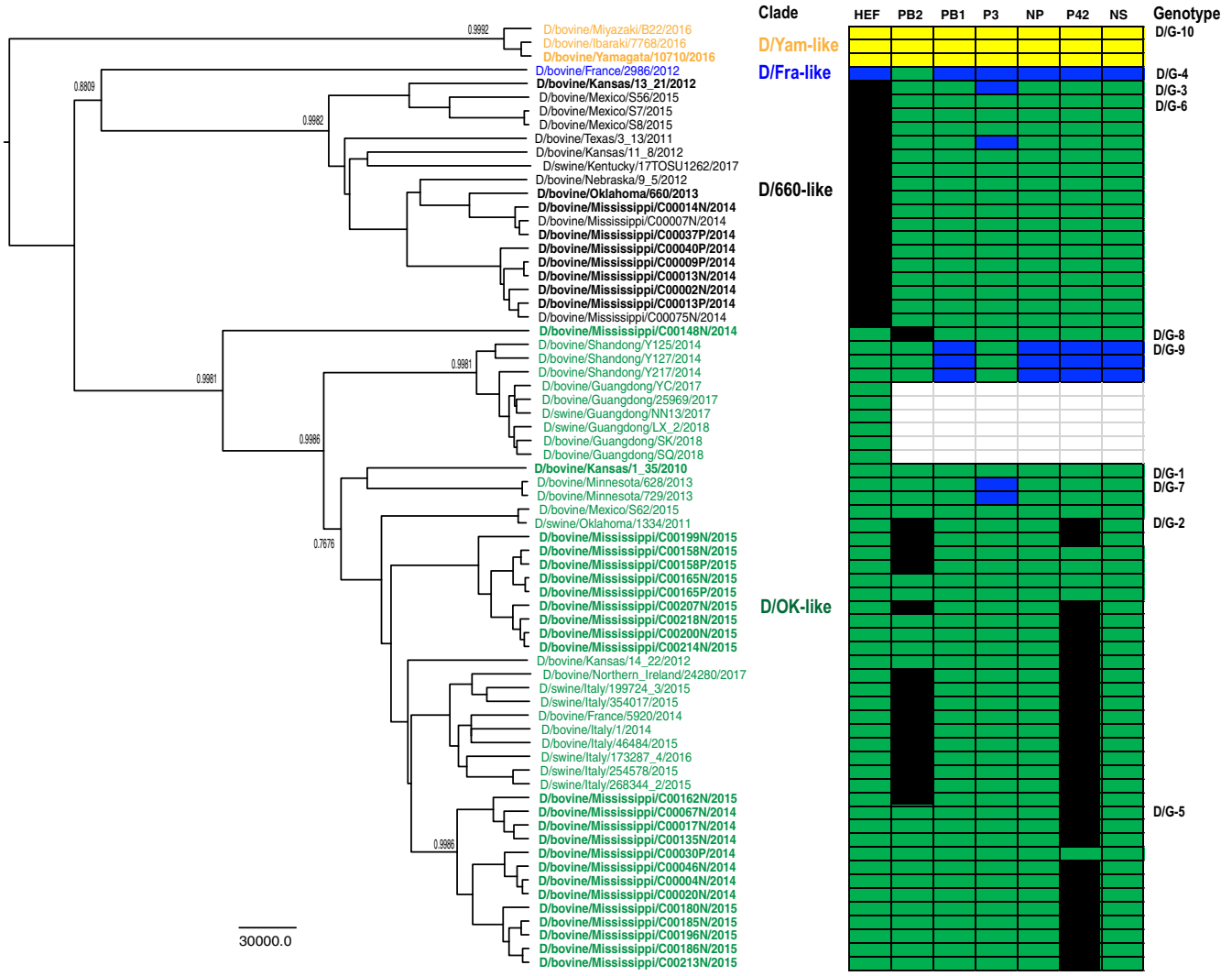

FIG 1 Phylogenetic analyses of influenza D viruses (IDVs). Multiple-sequence alignments (MSA) of gene sequences were generated using MAFFT v7.273 (30). Phylogenetic tree analyses were conducted using BEAST version 2.5 (31) with strict clock and HKY substitution model. Chain length was set to 50 million with a sample frequency of 5,000, and samples were combined with 10% burn-in removal using Log Combiner (https://beast.community/logcombiner). The maximum credibility trees were generated using Tree Annotator (https://www.beast2.org/treeannotator/), and posterior probability was determined as the robustness of the tree clades. Phylogenetic trees were then visualized using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). The bar indicates the scale for the mean height of each node in the tree to the mean height across the entire sample of trees for that clade. The genotype analysis was adapted from a prior study (32), and genetic clades were determined by a minimum posterior probability of 0.90 for tree clade credibility and a nucleotide sequence identity of ≥97.5%. In the colored genotypes, the uncolored segments indicate the segments were not available in this analysis. The viruses highlighted in boldface are used in antigenic analyses (Fig. 2).

Phylogenetic analyses suggested that there was a large genetic diversity among the internal genes of these IDVs and that 2, 3, 3, 3, 4, and 3 genetic clades were identified for PB2, PB1, P3, NP, P42, and NS gene segments of these IDVs, respectively. With the unique combination of genetic clades across 7 genetic segments of IDVs, 10 genotypes (D/G-1 through D/G-10) were identified that were distributed across North America, Europe, and Asia (Fig. 1). Multiple genotypes cocirculated in the same year, and at least six have been identified for at least 2 years (Fig. 2A). At the order-buyer facility we studied, active reassortment occurred and facilitated the emergence of new genotypes; five genotypes, D/G-1, D/G-2, D/G-5, D/G-6, and D/G-8, were identified in 2014 and 2015, and four of these genotypes were identified across at least two sampling periods within the same year or across 2 years (Fig. 2B).

To identify whether there were tissue-specific genetic features, we compared genomic sequences for three paired isolates from nasal swabs and nasopharyngeal

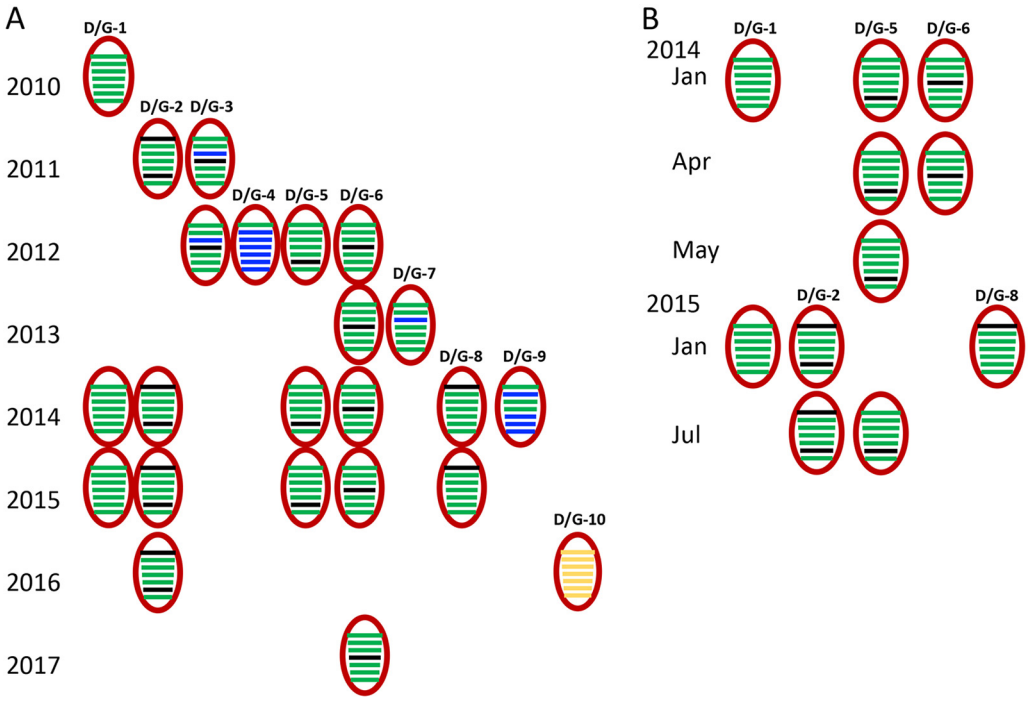

FIG 2 Temporal distribution of genetic reassortants of IDVs. (A) Temporal distribution of genotypes identified using the isolates from this study and those from the public database. (B) Temporal distribution of genotypes identified using the isolates from this study at a single order-buyer cattle facility. The segments in the oval figures are used to represent PB2, PB1, P3, HEF, NP, P42, and NS, and the colors indicate individual genetic clades, as shown in Fig. 1 and Fig. S1 in the supplemental material. The representative isolates for each genotype are also shown in Fig. 1.

swabs from the same animal. Results suggested that all seven gene segments from each pair of isolates had high sequence identities, ranging from 99.95% to 100%, and no particular mutations were identified between nasal swab-derived viruses and nasopharyngeal swab-derived viruses.

Antigenic diversity of IDVs. To test IDV antigenic diversity, cattle sera were generated against six representative IDVs (D/13N, D/46N, D/OK, D/660, D/bovine/Kansas/1-35/2010 [D/1-35], and D/Yam), representing three HEF genetic clades as described above (Fig. 1). The homologous HAI titers of these sera varied from 1:80 to 1:320. HAI assays were conducted on a total of 35 isolates against these six reference sera, and all isolates cross-reacted to all sera with titers ranging from 1:20 to 1:640. HAI titer-based antigenic cartography analysis showed that antigenic distances among these isolates were small, with the largest antigenic distances being only 2.0477 U (each unit is equal to a 2-fold change in HAI titer) (Fig. 3A and Table S2). However, despite short distances, the antigenic properties of the 10 D/OK-clade viruses were distinct from those of the 24 D/660-clade viruses. D/Yam from the D/Yam-like clade was antigenically similar to D/OK-clade viruses. The neutralization inhibition (NI) titer-based antigenic cartography analyses on 32 isolates showed patterns similar to those of the HAI data (Fig. 3B and Table S3). In summary, antigenic analyses using both HAI and NI data suggested that there were only small antigenic variations among IDVs and that these IDVs can be separated into two antigenic clusters, in which the viruses were correlated with those in two genetic clades (i.e., D/OK-like viruses and D/660-like viruses) in the phylogenetic analyses (Fig. 1).

Growth kinetics. Prior laboratory IDV infection studies in swine, cattle, and guinea pig demonstrated that IDV were detected in the upper and/or lower respiratory tract (1, 17–20). Due to the variations in temperatures across respiratory tracts in these animals, we aimed to evaluate the impact of temperature on growth kinetics of IDVs. The body temperatures were about 39°C and 38.6°C in swine and cattle, respectively. Thus, in this

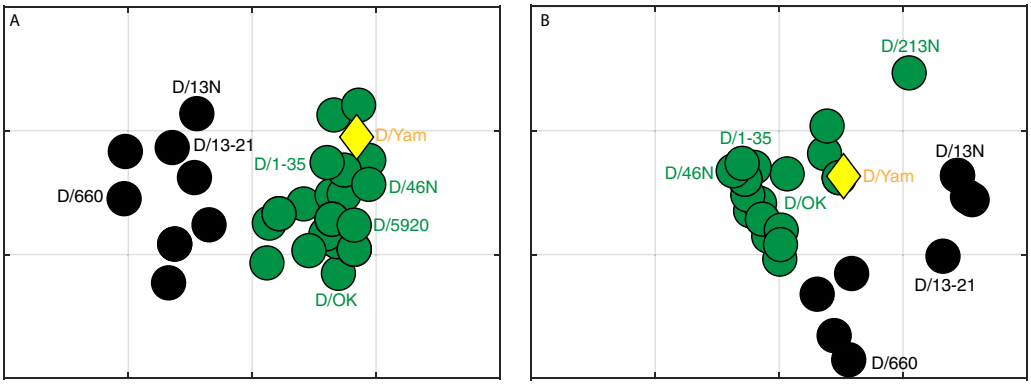

FIG 3 Antigenic maps for IDVs. (A) Antigenic map derived from HAI data using 35 IDV isolates. (B) Antigenic map derived from NI data using 30 IDV isolates. The bovine polyclonal sera against representative isolates from at least three genetic clades (D/OK-like viruses, D/OK, D/46N, and D/1-35; D/660-like viruses, D/660 and D/13N; and D/Yam-like virus, D/Yam) (Fig. 1) were used in generating both HAI (Table S2) and NI data (Table S3). Antigenic cartography was constructed on the basis of HAI data by using AntigenMap (http://sysbio.missouri.edu/AntigenMap) (33, 34). A titer of 10 was used as the low-reactor cutoff. The antigenic map utilized low-rank matrix completion to minimize the noise in the HAI or NI data and multiple dimensional scaling to generate the map reflecting the antigenic distances embedded in HAI or NI data. The *x* axis and *y* axis are exchangeable and used to define the antigenic distances among the antigens. Each unit in the antigenic map corresponded to a $\log_2$ unit in the HAI or NI titers.

study, we used three temperatures to mimic those across swine and cattle respiratory tracts, 33°C in upper respiratory tract (nasal turbinate), 37°C in middle respiratory tract (tracheal), and 39°C in lower respiratory tract (lung), and HRT-18 cells were used in the growth kinetics analyses. To evaluate the replication diversity among IDVs, 10 representative isolates from 7 different IDV genotypes (Fig. 1 and Fig. S1), including D/13-21, D/13N, D/bovine/Mississippi/C00199N/2015 (D/199N), D/1-35, D/bovine/Mississippi/C00148N/2014 (D/148N), D/10710, D/bovine/Mississippi/C00030P/2014 (D/30P), D/46N, D/bovine/Mississippi/C00165N/2015 (D/165N), and D/bovine/Mississippi/C00158N/2015 (D/158N), were selected. Results showed that all 10 isolates were able to replicate under the three conditions and had similar growth properties at 33°C and 37°C. However, with the exception of D/46N from genotype D/G-5 and D/199N from genotype D/G-2, virus growth was differentially impaired at 39°C; D/13N (genotype D/G-6), D/Yam (D/G-10), and D/13-21 (D/G-3) were most affected (Fig. 4).

Variable IDV pathogenicity in mice. To evaluate the pathogenicity of IDVs, six representative viruses covering different genotypes (D/46N, genotype D/G-5; D/bovine/Nebraska/9-5/2012 [D/Neb], D/G-6; D/13N, D/G-6; D/158N, D/G-8; D/148N, D/G-8; D/5920, D/G-2) were used to infect mice. These viruses had variations in the genetic clades of PB2, HEF, and/or P42 (Fig. 1). Mice were infected with 10^5 50% tissue culture infectious doses ($TCID_{50}$) of each virus, and clinical signs were recorded for 14 days. Mice did not lose weight and showed no clinical signs, and no mortality was observed after infection, irrespective of infecting virus.

Viral replication and tissue tropism were analyzed at 3, 5, and 7 days postinoculation (dpi) using quantitative reverse transcription-PCR (qRT-PCR). Similar patterns of viral replication were observed between groups, with peak titers seen at 5 dpi and virus still detected at 7 dpi. The highest viral titers were observed in the nasal turbinates (up to 8 $\log_{10}$ RNA copy/g of organ, depending on the strain) (Fig. 5). Viral replication was also observed in tracheas (up to 7.6 $\log_{10}$ RNA copy/g of organ) and lungs (up to 6.7 $\log_{10}$ RNA copy/g of organ) of infected mice. These results show that IDV can replicate robustly in the respiratory tract of mice, preferentially in the upper respiratory tract.

Viral RNA was also detected in nonrespiratory organs of infected mice, mainly at 3 and 5 dpi, with viral titers of up to 5.9 $\log_{10}$ RNA copy/g in liver and of up to 6.1 $\log_{10}$ RNA copy/g in intestines. Of note, D/158N was not able to replicate in the intestines of mice.

D/46N replicated to a lesser extent than other strains with D/OK-like HEF (D/5920 and D/158N), but the titers across different tissue samples are not statistically different

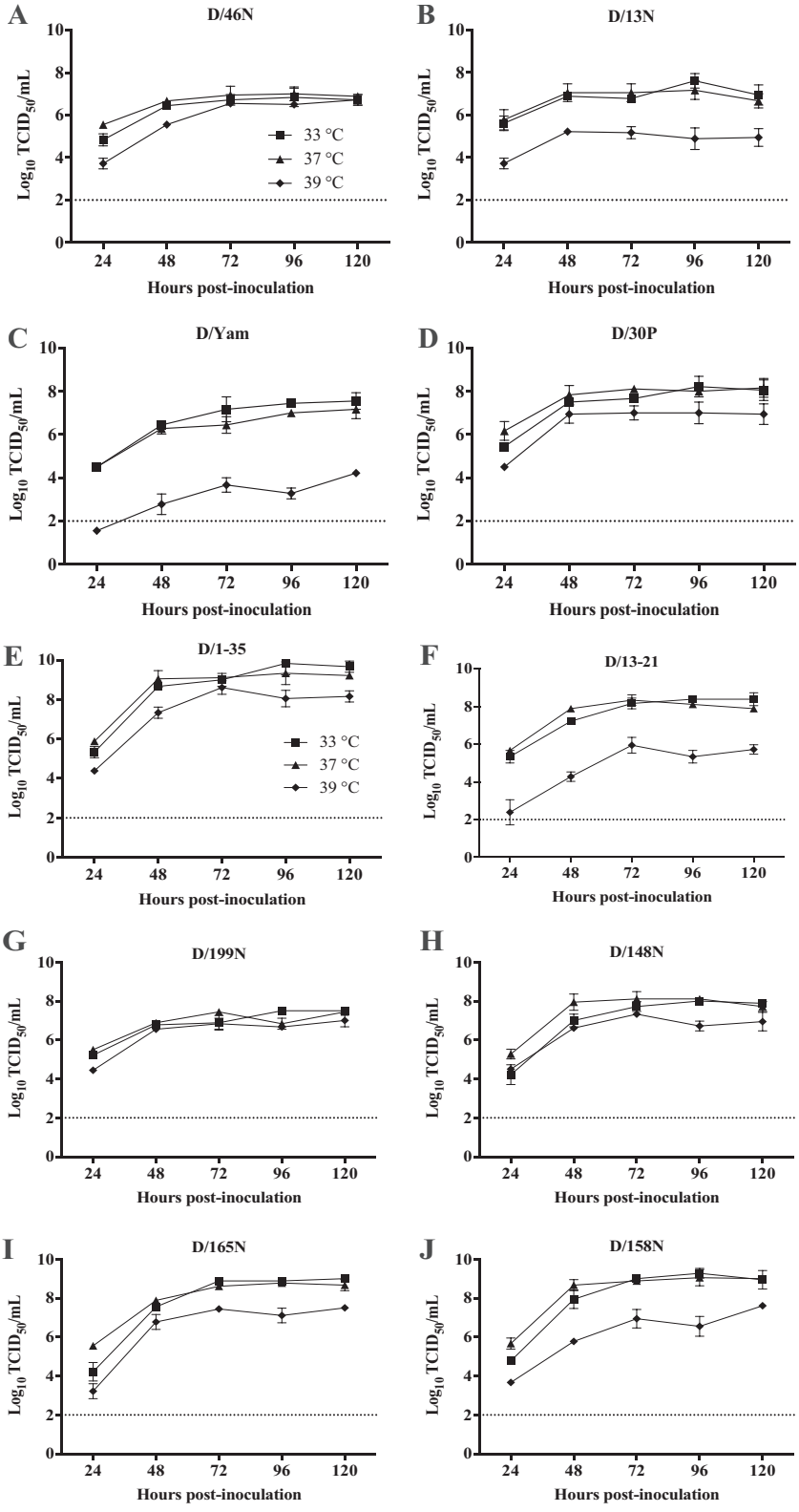

FIG 4 Growth kinetics of IDVs. Growth kinetics assays were carried out for four representative isolates, D/46N (A), D/13N (B), D/Yam (C), and D/30P (D), as well as D/1-35 (E), D/13-21 (F), D/199N (G), D/148N (H), D/165N (I), and D/158N (J), at 33°C, 37°C, and 39°C in triplicates at an MOI of 0.1. At each time point, medium was collected and replaced, and $TCID_{50}$ was determined for each sample taken. The average $TCID_{50}$/ml and standard deviations were calculated based on the $TCID_{50}$ results for each experimental replicate at each time, and the dotted line denotes the detection limit.

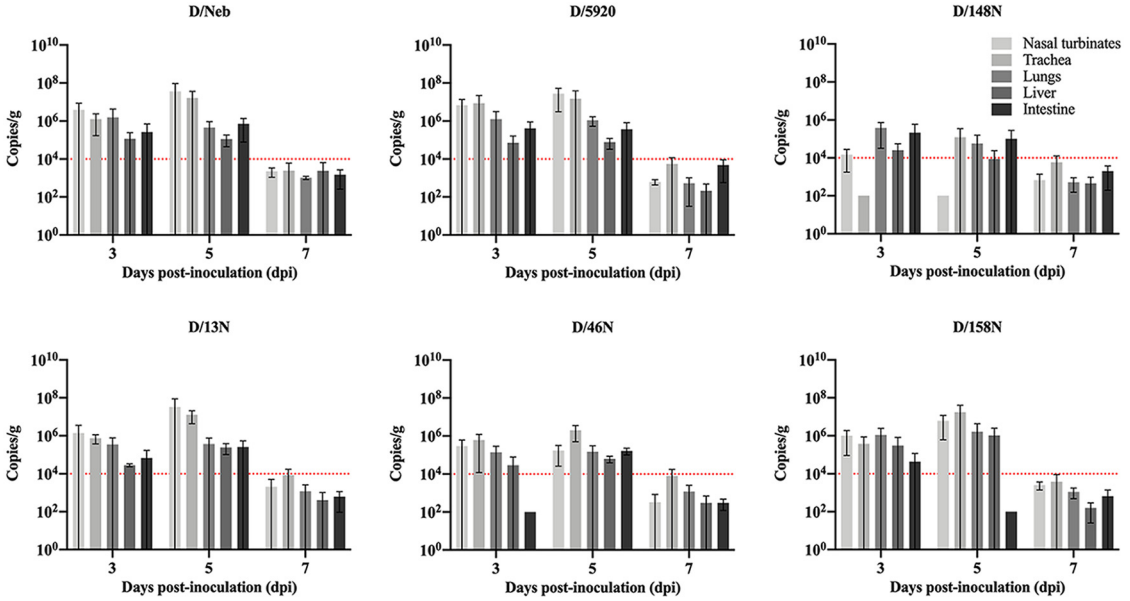

FIG 5 Pathogenesis of IDVs in mice. Each of six representative isolates, D/Neb, D/5920, D/148N, D/13N, D/46N, and D/158N, were inoculated into DBA/2JRj mice, 15 mice/isolate. Mice were infected intranasally with 10^5 $TCID_{50}$ of each virus. None of the experimental or control mice suffered loss of weight or showed any clinical signs. Viral replication in nasal turbinate, trachea, lungs, liver, and intestines was determined using quantitative RT-PCR, and the dotted line indicates the detection limit.

among these viruses. The virus was detected in similar organs but at lower titers than its counterparts, for example, 5.1 ($\pm$0.6 standard deviations) $\log_{10}$ RNA copy/g for D/46N and 7.3 ($\pm$0.5) $\log_{10}$ RNA copy/g for D/5920 in nasal turbinate tissue. However, the viral loads from the same tissue collected at either 3, 5, or 7 dpi were not statistically different between D/Neb and D/13N, both of which belong to the genotype D/G-6. Among six IDVs we tested, D/148N was the least efficient at replicating in mice (Fig. 5). Together, these results show an influence of viral genetic differences primarily on viral load in tissues.

Limited cross-protection against homologous or heterologous challenge of IDVs in cattle. Based on the data from our surveillance showing that IDVs were able to be isolated from seropositive cattle, we hypothesized that there would be limited cross-protection between IDVs. To test this hypothesis, we evaluated the cross-protection of two antigenically diverse isolates, D/13N and D/46N, through homologous and heterologous challenges of cattle. Our prior studies showed that both D/13N and D/46N can infect cattle and shed viruses (3, 17). The first challenge was performed at 0 dpi with D/13N, followed by the second challenge with either D/13N or D/46N at 22 dpi (Fig. 6).

The six cattle infected with D/13N shed virus from 1 to 8 dpi, and all cattle seroconverted at 7 dpi with homologous HAI titers ranging from 1:80 to 1:160 at 21 dpi, just prior to rechallenge (Fig. 6).

After the second challenge, the three cattle in the homologous challenge group shed virus at 23 to 24 dpi, and their serum HAI titers increased throughout the course of the study, peaking at 36 dpi at 1:640 to 1:1,280. The three cattle in the heterologous challenge group shed virus at 23 dpi to 26 dpi, and their serum titers increased throughout the course of the study, peaking at 29 dpi at 1:640 to 1:1,280 for both D/13N and D/46N (Fig. 6). Overall, the replication of the heterologous virus was more robust than that of the homologous virus upon rechallenge.

Two mock control cattle inoculated with phosphate-buffered saline (PBS) and challenged with D/46N at 22 dpi shed viruses from 23 dpi to 28 dpi and seroconverted at 29 dpi following virus inoculation of D/46N at 22 dpi (Fig. 6), and two negative-control cattle did not shed any viruses and remained seronegative against IDVs throughout the study (data not shown).

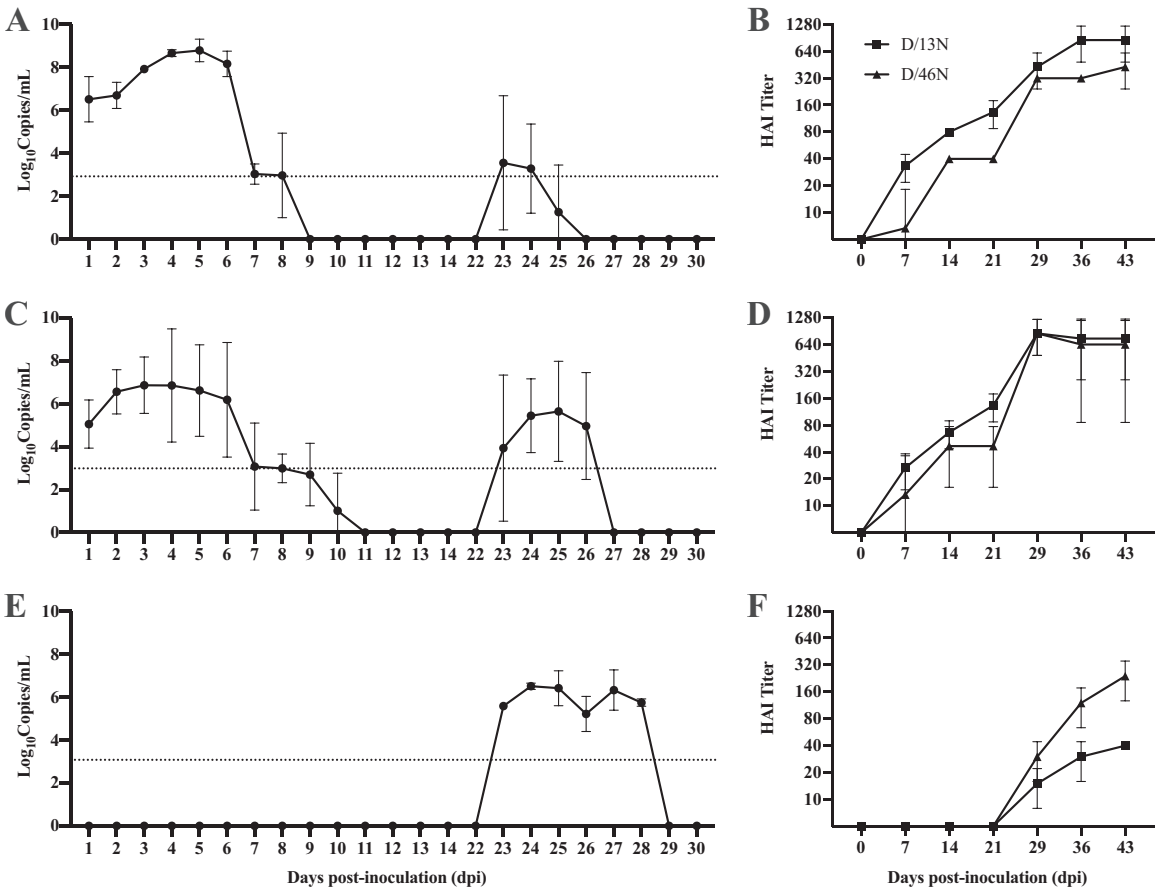

FIG 6 Cross-protection of IDVs from the immunity generated by prior exposures through homologous and heterologous challenges of influenza D viruses. (A and B) The viral shed (A) and serological responses (B) in the homologous challenge group, in which three cows were infected with D/13N and rechallenged with the same virus D/13N at 22 dpi. (C and D) The viral shed (C) and serological responses (D) in the heterologous challenge group, in which three cows were infected with D/13N and rechallenged with a different virus D/46N at 22 dpi. (E and F) The viral shed (E) and serological responses (F) in the mock control group, in which three cows were inoculated with PBS and challenged with D/46N at 22 dpi. The copies of IDVs were determined using quantitative RT-PCR, and the dotted line denotes the detection limit. The serological titers were determined using HAI assays, each serum sample was tested against D/13N and D/46N in triplicates, and the $\log_2$ HAI titers were averaged and a standard deviation was calculated.

DISCUSSION

To better understand the evolutionary relationship of IDVs identified worldwide, we collected all publicly available sequence data and generated additional genomes ourselves. Phylogenetically the IDV HEF genes can be grouped into four clades, D/OK-like, D/660-like, D/Yam-like, and D/Fra-like. Two of these clades were geographically defined (i.e., D/Yam-like from Asia and D/Fra-like from Europe), whereas the other two (D/OK-like and D/660-like) had wider distribution and are present not only in North America but also in Eurasia (Fig. 1). Similar observations were also shown in internal gene phylogenies. Genotypic analyses showed that active reassortment occurs globally and also within a single cattle facility (Fig. 2). This is consistent with an earlier report that showed reassortants between D/OK-like and D/660-like viruses (15). Some of the identified genotypes were maintained for at least a year, and multiple genotypes were present at the same time. The latter point suggests that an emerging genotype does not necessarily sweep through and predominate the population; instead, multiple genotypes are likely to cocirculate in the population. Such an evolutionary pattern seems to be different from that of influenza A and B viruses, where an emerging reassortant tends to predominate in human or animal populations in a selective sweep (26). How widespread this pattern of IDV circulation is remains to be determined, as there is limited global representation in genetic databases.

Despite a level of genotypic variation, there was considerable antigenic similarity between IDV isolates. There was a high level of cross-reactivity between viruses and cattle polyclonal sera generated against six strains of IDVs, representing three of four identified HEF clades. Nevertheless, two antigenic clusters could be defined with both isolates from Eurasia, with D/5920, D/OK, and D/Yam clustering together in one (Fig. 3). During virologic surveillance, IDV isolates could be recovered from 13 cattle with HI titers of $\geq$1:10 (see Table S1 in the supplemental material), suggesting that prior IDV infection does not provide sterilizing immunity. This hypothesis was further validated by homologous and heterologous challenge studies in cattle, where both viruses could replicate upon rechallenge. The cattle with prior exposure to homologous challenge did, however, have lower viral loads (titers and duration) than those experiencing heterologous challenge (Fig. 6). These results could partially explain why, although they are panzootic in various animal hosts worldwide, IDVs display a limited level of antigenic drift.

Serological analyses suggested that the majority of cattle had a higher titer to one of two testing viruses, D/46N or D/13N. However, in the same lot with the cattle upon arrival, some cattle showed seropositivity against D/46N (and seronegative to D/13N), whereas others had seropositivity against D/13N (and seronegative to D/46N); similar observations were shown in the cattle across different lots (data not shown). Thus, it is likely the herd in the same lot had been exposed to the viruses from both antigenic clusters before being brought to the facility. However, due to the lack of the detailed geographic origin for individual cattle and the limited number of samples we collected from the herds, we were not able to perform epidemiological analyses to illustrate the geographic distribution of IDV seroprevalence in cattle farms, presenting a limitation of this study. In this same facility, genetic and antigenic analyses suggested both D/OK-like and D/660-like viruses were enzootic, and reassortment events were also documented. In the future, a more systematic study needs to be designed to test the hypothesis that the dynamics of antibody titers were caused by the prevalence dynamics of D/OK-like or D/660-like viruses and an alternative hypothesis that the dynamics of antibody titers could have driven the predominance of D/OK-like or D/660-like viruses in this facility.

Prior studies suggested that IDVs were present in the upper respiratory tracts of cattle (17), suggesting potential biases in tissue tropism. To test the effects of variations of temperatures through the respiratory tract on viral replication, we evaluated the growth kinetics of 10 selected strains. With the exception of D/46N and D/199N, the replication efficiency of the viruses we tested was lower at 39°C than at 33 and 37°C, indicating most IDVs would replicate better at temperatures associated with the upper respiratory tract. This was universally true, and some IDVs might have an enhanced ability to replicate at higher temperatures and cause lower respiratory tract disease. Such variation could be a factor to be considered when an IDV isolate is selected for a pathogenesis study.

In this study, to broaden the phenotypic diversity of IDVs, we attempted to include isolates from multiple genotypes in the phenotypic analyses, including antigenic analyses, growth kinetics, and pathogenesis analyses. With different extents of phenotypic diversity, it is clear that molecular diversity among these strains affected phenotypic diversity, but no apparent patterns (e.g., certain genetic clades for a specific gene segment) were identified to be associated with specific phenotypes. It is, however, interesting that the viruses in the D/OK-like HEF clade seem to bear more phenotypic diversity than those in the D/660-like clade. In IDV, the surface glycoprotein HEF is responsible for receptor binding, receptor cleavage, and membrane fusion (27). Receptor binding evidence suggested that IDV, like ICV, binds preferentially to the 9-*O*-acetylated sialic acid, but unlike ICV, IDV has the capacity to bind and interact with a wider range of glycan moieties due to an open receptor conformation (27). This open receptor binding pocket has been suggested to explain the broader cellular and host tropism noted in IDV; however, equally notable with regard to tropism is IDV's robust resistance to low-pH inactivation and high temperature stability compared to that of influenza A virus (IAV), influenza B virus (IBV), and ICV (28). Fourteen amino acids of the HEF protein were shown to be different between the D/660-like isolates

and the D/OK-like isolates: V68I, A80D, K181T, T188A, R212K, A215V, A252T, L256F, G288N, K312R, I469V, S486N, N627K, and F654S. Their respective role in determining virus phenotype is unknown.

In summary, this study suggests that limited protection afforded by preexisting immunity against IDVs in cattle herds, as well as the circulation of at least two different antigenic clades of viruses, likely facilitate the high prevalence of IDVs in animal populations.

MATERIALS AND METHODS

Viral and serologic sampling. The nasal/nasopharyngeal swabs and serum samples were collected as described elsewhere (3). Briefly, nasal or nasopharyngeal swabs were collected from cattle either upon arrival or at the animal hospital of an order-buyer facility in Mississippi using Starswabs II (Starplex Scientific, Cleveland, TN) and 33-inch double guarded culture swabs (Santa Cruz Animal Health, Dallas, TX), respectively. Blood was collected from the jugular vein using disposable 18-gauge needles and blood tubes under vacuum, and serum was separated via centrifugation. Swabs were transported in 1 ml of Media 199 (Gibco Life Technologies, Grand Island, NY) with 1% penicillin-streptomycin (PenStrep; Gibco Life Technologies) in 4-ml Corning cryogenic vials (Corning, Tewksbury, MA). Swabs were stored at −80°C, and sera were stored at −20°C.

Cells. Maintenance of human rectal tumor cells (HRT-18G) (ATCC, Manassas, VA) and IDV infections were carried out as described elsewhere (17). Briefly, cells were maintained at 37°C with 5% CO_2 using 1× Dulbecco's modified Eagle's medium (DMEM) with 1% PenStrep and 5% fetal bovine serum (FBS) (Gibco Life Technologies, Carlsbad, CA) prior to viral infection. Respiratory swabs collected that had been determined positive by qRT-PCR were used to inoculate HRT-18G cells. Cellular infections were carried out using Opti-MEM (Gibco Life Technologies) media with 1% PenStrep, 0.2% bovine serum albumin (BSA; Gibco Life Technologies), and 0.5 μg/ml TPCK-trypsin (Gibco Life Technologies). Infected cells were incubated for 5 dpi, and swab material was serially passaged (up to five passages) until the hemagglutination (HA) assay was positive. Cells were washed three times in phosphate-buffered saline (PBS). Any passage that demonstrated fungal contamination was filter sterilized before being passaged again.

HA, HAI, and NI assays. HA assays were performed using either 0.5% turkey erythrocytes with incubation at 4°C or 1% horse erythrocytes with incubation at 37°C. Briefly, using a U-bottom 96-well plate (USA Scientific, Ocala, FL), virus was serially diluted from 1:2 to 1:2,048 in PBS prior to being incubated for 45 min in an equal volume of 0.5% turkey erythrocytes (Lampire Biological Laboratory, Pipersville, PA) or 1% horse erythrocytes (ENVT Equestrian Center, Toulouse, France). HA titers were determined based on the highest dilution of complete agglutination.

To determine the anti-IDV antibody titers in cattle serum, serum samples were treated for at least 18 h at 37°C in 1:3 receptor-destroying enzyme (RDE) (Denka Seiken Ca., Tokyo, Japan). RDE-treated serum was heat inactivated at 55°C for 30 min before being adjusted to a 1:10 dilution using PBS. The 1:10 RDE-treated sera were serially diluted in U-bottom 96-well plates from 1:10 to 1:1,280 and incubated with equal volumes of 8 HA units (final concentration of 4 HA units) for 45 min at room temperature. Finally, each well was combined with an equal volume of 0.5% turkey erythrocytes and incubated for 45 min at 4°C. HAI titers in each serum were determined based on the highest titer that completely inhibited agglutination; samples with an HAI titer of ≥1:40 were determined to be seropositive against the testing virus as described elsewhere (3, 25).

To determine the anti-IDV antibody titers in mouse sera, sera were treated with RDE and then adsorbed on packed horse erythrocytes. HAI assays were performed with 1% horse erythrocytes as previously described (5) against D/bovine/France/5920/2014 (D/5920), D/bovine/Nebraska/9-5/2012 (D/9-5) D/13N, D/bovine/Mississippi/C00148N/2014 (D/148N), and D/bovine/Mississippi/C00158N/2015 (D/158N). Mouse sera with HAI titers of ≥1:20 were considered IDV seropositive.

NI assays were performed by serially diluting serum and combining serum dilutions with equal volumes of 100 $TCID_{50}$ of virus prior to incubation at 37°C for 1 h. Each dilution was placed in a 96-well cell culture plate seeded with HRT-18G cells and incubated for 5 days at 37°C with 5% CO_2. Following incubation, the wells of the plate were transferred to a U-bottom 96-well plate and combined with equal volumes of 0.5% turkey erythrocytes before incubation for 45 min at 4°C. NI titers were determined by the highest titer of complete inhibition of agglutination.

RNA extraction and qRT-PCR. Viral RNA was extracted using a GeneJET viral DNA/RNA purification kit (Thermo Scientific, Pittsburgh, PA) or QIAamp viral RNA minikit (Qiagen) according to the manufacturer's protocol. The copies of viral loads were quantified using qRT-PCR using TaqMan fast virus 1-step master mix (Life Technology, Carlsbad, CA) or the QuantiNova probe RT-PCR (Qiagen) using the previously described PB1-specific primer set (1). The standard curve was established with the equation $\log_{10}$ (copy number of PB1 genes) = −0.33 (ΔC_T) + 14.265 ($R^2 = 0.9929$; $P < 0.001$), as described elsewhere (17). The standard curve was included in each analysis to standardize viral quantities across samples. The reaction was run on a QuantStudio 6 Flex real-time PCR system or a LightCycler 96. A positivity threshold was set at 3 $\log_{10}$ cDNA copy/ml for nasal swabs.

To quantify viral replication in mice, viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen). Real-time RT-PCR was carried out using the QuantiNova probe RT-PCR (Qiagen) and the primers and probe described elsewhere (1). The reaction was run on a LightCycler 96 and analyzed with LightCycler 96 (v1.1.0.1320) software (Roche). A positivity threshold was set at 4 $\log_{10}$ cDNA copy/g of organs.

Genomic sequencing. Sanger sequencing was carried out at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. vRNA was extracted using a GeneJET viral DNA/RNA purification kit, and reverse transcription and PCR amplification were carried out using influenza-specific primers as previously described (29). Amplified DNA template was sequenced by rhodamine or dRhodamine dye terminator cycle sequencing ready reaction kits with AmpliTaqDNA polymerase FS (Perkin-Elmer Applied Biosystems), and samples were analyzed on an Applied Biosystems 3730XL DNA Analyzer (PerkinElmer Applied Biosystems, Waltham, MA).

$TCID_{50}$. $TCID_{50}$ was determined as previously described (17). Briefly, viral samples were serially diluted from $1:10^1$ to, at most, $1:10^{12}$ in the viral infection media described above. Two hundred microliters of serially diluted virus was placed in four wells of HRT-18G-seeded 96-well plates for each dilution and incubated at 37°C in 5% CO_2 for 5 days. Following incubation, 50 μl of virally infected media from each well was transported to a U-bottom 96-well plate and incubated for 45 min at 4°C with an equal volume of 0.5% turkey erythrocytes.

Growth kinetics. HRT-18G cells were seeded in 6-well plates and incubated overnight at 37°C, 5% CO_2. The cells were washed with PBS and inoculated with IDV in 1 ml of Opti-MEM at a multiplicity of infection (MOI) of 0.1. After 1 h of incubation at 37°C, 5% CO_2, the inoculum was replaced with fresh infection medium; samples were collected at 24, 48, 72, 96, and 120 h postincubation to test their viral titers using $TCID_{50}$ in HRT-18G cells. To simulate the growth conditions at different locations in the respiratory tracts, the growth kinetics were determined for each virus at 33°C, 37°C, and 39°C. Each analysis was performed in triplicate.

Animal experiments. (i) Animals. Six-week-old female DBA/2JRj mice were purchased from Janvier LABS (Le Genest-Saint-Isle, France). The DBA/2 mice were housed at the Veterinary School of Toulouse in animal biosafety level 2 (ABSL-2) facilities and had food and water *ad libitum*. Experiments were conducted in accordance with European and French legislation on laboratory animal care and use (French Decree 2001-464 and European Directive CEE86/609), and the animal protocol was approved by the Ethics Committee "Sciences et santé animale," committee number 115 (protocol no. 2018030212288103).

Four-month-old healthy male dairy calves were purchased from the dairy operation at the Mississippi State University. All calves were determined to be seronegative for D/bovine/Mississippi/C00046N/2014 (abbreviated as D/46N), D/bovine/Mississippi/C00013N/2014 (D/13N), D/swine/Oklahoma/1334/2011 (D/OK), D/bovine/Oklahoma/660/2013 (D/660), D/bovine/Yamagata/10710/2016 (D/10710), and D/bovine/Kansas/1-35/2010 (D/1-35) using HAI assays. Cattle experiments were conducted under BSL-2 conditions, in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Mississippi State University (IACUC number 15-015 and number 16-634).

(ii) Generation of polyclonal sera in cattle. Each of four cattle was placed in a separate ABSL-2 room. After 7 days of acclimation, the four calves were given 10^7 $TCID_{50}$ of virus in a 10-ml dose of either D/1334, D/660, D/10710, or D/1-35. Serum was collected at 21 dpi. All sera were aliquoted and kept at −20°C prior to use.

(iii) Pathogenesis of IDV in mice. Six-week-old female DBA/2JRj mice were used, with 15 mice per IDV strain. Mice were separated into two groups: 3 noninfected (controls) and 12 infected. Mice were lightly anesthetized with a ketamine-xylazine combination (100 mg/kg and 10 mg/kg) and infected with 10^5 $TCID_{50}$ of each virus (in a volume of 30 μl per mouse) intranasally or with PBS as a control. Clinical observations were recorded daily, and weight was measured every 2 days. Any animal showing a weight loss greater than 30% or signs of suffering was humanely euthanized. Necropsies were performed at 3, 5, and 7 dpi (3 infected mice per day per IDV strain), and nasal turbinates, trachea, lungs, liver, and intestine (duodenum, jejunum, and colon) were sampled. Whole organs were dissociated using the tissue homogenizer Precellys 24 (Ozyme), resuspended in 500 μl of PBS, centrifuged, and stored at −80°C until further analysis. Blood was also collected at 14 dpi from all the remaining mice, which were then euthanized at 14 dpi.

(iv) Cross-protection of IDVs in cattle. To assess the cross-protection of IDVs across genetic clades, two prototype viruses, D/46N and D/13N, were selected. A total of eight calves were placed in three experimental groups: homologous challenge group ($n = 3$), heterologous challenge group ($n = 3$), and control group ($n = 2$). For the homologous challenge group, three calves were inoculated intranasally with 10^7 $TCID_{50}$ of D/13N in a volume of 10 ml at day 0 and then inoculated intranasally with the same amount of D/13N at 22 dpi; for the heterologous challenge group, three calves were inoculated intranasally with 10^7 $TCID_{50}$ of D/13N in a volume of 10 ml on day 0 and then inoculated intranasally with the same amount of D/46N at 22 dpi; for the control group, three calves were inoculated intranasally with 10 ml sterile PBS on day 0 and then inoculated intranasally with the same amount of D/46N at 22 dpi. All animals were euthanized at 36 dpi. Nasal swabs were taken each day from all calves to monitor viral shedding. Blood samples for serum isolation were collected at 0, 7, 14, 21, 29, and 36 dpi.

Genomic sequences from public databases. The unique HEF and genomic sequences were downloaded from Influenza Virus Resources (https://www.ncbi.nlm.nih.gov/genomes/FLU/) and Influenza Research Database (https://www.fludb.org/) on 3 October 2019. Only the viruses with a complete HEF gene segment were included in the phylogenetic analyses, and only those IDVs with the genomic sequences of all seven gene segments were included in the genotype analyses.

MSA, phylogenetic analysis, and genotype analyses. Multiple-sequence alignments (MSA) of gene sequences were generated using MAFFT v7.273 (30). Phylogenetic tree analyses were conducted using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) version 2.5 (31) with a strict clock and HKY substitution model. Chain length was set to 50 million with a sample frequency of 5,000, and samples

were combined with 10% burn-in removal using Log Combiner (https://beast.community/logcombiner). The maximum credibility trees were generated using Tree Annotator (https://www.beast2.org/treeannotator/), and posterior probability was determined as the robustness of the tree clades. Phylogenetic trees were then visualized using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). The genotype analysis was adapted from a prior study (32). The genotype for a specific IDV was determined by using a unique combination of genetic clusters for each segment of the IDV. For each gene segment, genetic clusters were determined by a minimum posterior probability of 0.90 for tree clade credibility and a nucleotide sequence identity of ≥97.5%.

Antigenic cartography. HAI and NI assays were performed for selected IDV isolates (Table 1) using the previously described bovine sera anti-D/46N, anti-D/13N, anti-D/OK, anti-D/660, anti-D/10710, and anti-D/1-35. D/bovine/Mississippi/C00180N/2015 (D/180N), D/bovine/Mississippi/C00004N/2014 (D/4N), D/bovine/France/5920/2014 (D/5920), and D/bovine/Mississippi/C00067N/2014 (D/67N) were used in HAI but not NI assays because of the difficulty in propagating those viruses. Antigenic cartography was constructed on the basis of HAI data by using AntigenMap (http://sysbio.missouri.edu/AntigenMap) (33, 34). A titer of 10 was used as the low-reactor cutoff. The antigenic map utilized low-rank matrix completion to minimize the noise in the HAI or NI data and multiple dimensional scaling to generate the map reflecting the antigenic distances embedded in HAI or NI data. Each unit in the antigenic map corresponds to a $\log_2$ unit in HAI or NI titers.

Statistical analyses. The nonparametric method of the Mann-Whitney test implemented in Prism (GraphPad, San Diego, CA) was used to compare the seroprevalence rates and the geometric mean titer of HAI titers between sick and healthy cattle. A 2-way analysis of variance test with Bonferroni's multiple-comparison test implemented in Prism (GraphPad, San Diego, CA) was used to evaluate viral loads in nasal washes or organs in mice. A P value of 0.05 was determined to be statistically significant for all analyses.

Data availability. Sequences were deposited in GenBank under accession numbers MT511333 to MT511546.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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